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Characterization of Virulence Plasmids and Plasmid-Associated Outer Membrane Proteins in *Shigella flexneri*, *Shigella sonnei*, and *Escherichia coli*

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The 140-megadalton plasmids of *Shigella flexneri* serotypes 1, 3, and 5, in addition to the 120-megadalton plasmid of *Shigella sonnei*, are associated with virulence. The present study showed that a 140-megadalton plasmid is also associated with virulence in *Escherichia coli*. When these plasmids were cleaved with *Eco*RI or *Bam*HI restriction endonucleases, considerable homology was evident in plasmids from *S. sonnei* strains, whereas only a few common fragments were observed among the *S. flexneri* and enteroinvasive *E. coli* plasmids. Nitrocellulose filter hybridization demonstrated that, despite variations in restriction sites, all these plasmids shared a considerable complement of homologous sequences. Minicell-producing strains were obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis. Transmission electron microscopy of infected HeLa cells showed that minicells from invasive strains retained the invasive phenotype. Sixteen polypeptides were labeled when *S. flexneri* 5 minicells were incubated with [^{35}S]methionine. Fourteen of these plasmid-coded polypeptides were associated with the outer membrane in invasive strains of *S. flexneri* 5, and nine polypeptides of similar molecular weight were labeled in the outer membrane of invasive strains of *S. flexneri* 3, *S. sonnei*, and *E. coli*. Seven of the *S. flexneri* 5 polypeptides were not labeled in a noninvasive strain which had sustained a large deletion in the virulence-associated plasmid, and none were labeled in minicells which no longer harbored this plasmid.

The pathogenic potential of bacteria belonging to the genus *Shigella* is correlated with the ability of these organisms to invade and multiply within cells of the colonic epithelium. Early genetic studies dealt with chromosomal loci which were necessary for virulence in this genus (3, 4, 7); however, more recent work has shown that extrachromosomal elements are also required for virulence in both *Shigella sonnei* (11, 17, 19) and *Shigella flexneri* (20). In the former species, a 120-megadalton (Mdal) plasmid is necessary for the expression of the form I antigen consisting of O-polysaccharide side chains on the lipopolysaccharide (LPS). The latter species harbors a 140 Mdal plasmid which is not involved in LPS expression. The assertion that these large plasmids are associated with a virulent phenotype is based upon the following observations: (i) a large plasmid is invariably present in virulent shigella strains; (ii) loss of a large plasmid is always accompanied by conversion to avirulence; and (iii) virulence is reconstituted in some plasmid-free strains upon reacquisition of a large plasmid from the homologous species.

In the present study, the association of extrachromosomal elements with virulence in invasive enteric pathogens was extended to include a "shigella-like," enteroinvasive *Escherichia coli* strain. In addition, the degree of homology shared by the virulence-associated plasmids of *S. flexneri*, *S. sonnei*, and *E. coli* was analyzed by restriction endonuclease digestion and nitrocellulose filter DNA blot hybridization. Finally, the biosynthetic activity of these plasmids was studied in anucleate minicells, and several plasmid-associated polypeptides were identified in the bacterial outer membrane.

Preliminary results of this study have been presented previously (T. L. Hale, P. J. Sanson-

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MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids and bacterial strains used are listed in Table 1.

Media and cultural conditions. Bacteria were routinely grown in 37°C in Penassay (PA) or brain heart infusion broth or on Trypticase Soy agar. Antibiotics were used at the following final concentrations: kanamycin, 20 μg/ml; tetracycline, 20 μg/ml; nalidixic acid, 50 μg/ml.

Antibiotic sensitivity tests. BBL Sensi-Discs (BBL Microbiology Systems, Cockeysville, Md.) were routinely used for antibiotic susceptibility testing on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.).

Serological tests. Typing was performed by slide agglutination tests with isolated colonies and specific rabbit antisera. *S. flexneri* strains were verified by using antisera specific for group and type antigens. *S. sonnei* form I and form II colonies, initially identified by low-power stereomicroscopy, were verified by form I and form II specific antisera.

Virulence assays. The Sereny test (23) and HeLa cell invasion (8) were used as reference assays for virulence. In the former, 1 drop of a bacterial suspension, containing approximately 5.0×10^9 organisms, was inoculated into the conjunctival sac of a guinea pig. Strains which elicited keratoconjunctivitis within 3 days were considered virulent. Invasiveness was also routinely assayed by tissue culture infection. Nonconfluent monolayers of HeLa cells were inoculated with bacteria and incubated for 2 h at 37°C, and Giemsa-stained preparations were examined microscopically.

Isolation of avirulent mutants. Old cultures of invasive *E. coli* 4608-58, which had been kept on Trypticase Soy agar slants for several months, were streaked onto Trypticase Soy agar medium. Isolated colonies were assayed for virulence. When avirulent colonies were identified, plasmid DNA was compared with that of virulent colonies. Isolation of avirulent variants of *S. flexneri* M25-8 and M90T has been described in a previous paper (20).

Plasmid transfer. Purified donor and recipient cells were grown at 37°C in PA broth to midexponential phase, and 0.2 ml each of the donor and recipient cultures was mixed in 10 ml of PA broth. After overnight incubation, appropriate dilutions were plated on selective medium.

Plasmid DNA isolation. Rapid plasmid detection was performed according to the procedure of Kado and Liu (10) with minor modifications. Tracking dye (5 μl of 0.07% bromophenol blue, 7% sodium dodecyl sulfate [SDS], and 33% glycerol) was added to 20 μl of plasmid DNA, and these samples were applied to a 0.7% agarose gel prepared with E buffer (40 mM Tris–2 mM EDTA, adjusted to pH 7.9 with acetic acid). Agarose gels were electrophoresed for 2 h at 90 V and stained for photography as previously described (11).

Large quantities of plasmid DNA for restriction analysis were isolated by an adaptation of the above procedure (10). Stationary-phase, 250-ml PA cultures were washed in E buffer and lysed by incubation for 1 h at 65°C in 50 mM Tris-hydrochloride buffer with 3% SDS (pH 12.6). The lysate was neutralized with 2.0 M Tris-hydrochloride buffer (pH 7.0) and centrifuged at 15,000 × *g* for 15 min. The supernatant was collected and mixed with $CsCl_2$ and ethidium bromide and then centrifuged for 40 h at 100,000 × *g*; the plasmid DNA band was then collected. The ethidium bromide was extracted with isopropanol saturated with $CsCl_2$, and the DNA solution was dialyzed against E buffer. The DNA was recovered by ethanol precipitation, frozen in dry ice, and centrifuged at 15,000 × *g* for 20 min. The resulting pellet of DNA was suspended in E buffer and stored at 4°C.

Restriction endonuclease analysis. Purified plasmid DNA (4 to 8 μg), suspended in E buffer, was mixed with 5 μl of *Eco*RI or *Bam*HI restriction endonuclease (New England Biolabs, Beverly, Mass.) in a final volume of 50 μl. This mixture was incubated at 37°C for 120 min, heated to 65°C for 5 min, and cooled to 4°C. Stop mix (5 μl of 0.07% bromophenol blue, 7% SDS, and 2% Ficoll) was added, and the entire mixture was applied to a 0.7% vertical agarose gel prepared with Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA, 89 mM boric acid, pH 8). The DNA fragments were electrophoresed at 30 V for 12 h, stained, and photographed as described previously (11).

Nitrocellulose filter hybridization. Hybridization on nitrocellulose filters was performed under stringent conditions, according to Southern's methodology (24) with modifications. *Eco*RI restriction fragments were separated electrophoretically and depurinated by immersing the 0.7% agarose gel in 0.25 M HCl for 10 min. The DNA was denatured by two 15-min immersions in 1.5 M NaCl–0.5 N NaOH and neutralized by two 30-min immersions in 3 M NaCl–0.5 M Tri-hydrochloride (pH 7.0). The DNA was then transferred overnight onto a nitrocellulose filter along a 20× to 2× SSC gradient (2× SSC is 0.15 M NaCl–0.05 M sodium citrate). The filter was rinsed in 2× SSC for 20 min, air dried, and baked for 3 h at 80°C. Plasmid DNA from *S. flexneri* M90T was radiolabeled by nick-translation, using ^{32}P-deoxyribonucleotides achieving a specific activity of 2.5×10^7 cpm/μg of DNA. The nitrocellulose filter was prehybridized by immersion for at least 4 h in a solution of 40% formamide, 5× SSC, 5× Denhardt solution, 50 mM sodium phosphate (pH 6.5), and heat-denatured calf thymus DNA at 100 μg/ml. Specific hybridization was carried out in a sealed plastic bag at 42°C for 17 h in 10 ml of the following solution: 50% formamide, 5× SSC, 1× Denhardt solution, 20 mM sodium phosphate (pH 6.5), 100 μg of heat-denatured calf thymus DNA per ml, and 5×10^6 cpm of radioactive M90T plasmid DNA. The filter was then washed three times with 250 ml of 2× SSC–0.1% SDS for 5 min and twice with 250 ml of 0.1× SSC–0.1% SDS at 50°C for 30 min. The washed filter was air dried and exposed overnight to a Kodak XAR_5 film at −80°C.

Mutagenesis and minicell purification. Minicell-producing mutants were isolated after mutagenesis of parental strains with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Gemski and Griffin (6). Purification of free minicells from 18-h PA broth cultures was accomplished by differential centrifugation and two sucrose density gradient separations (6). Purified minicell suspensions usually contained less

TABLE 1. Bacterial strains and plasmids

Strain	Serotype	Relevant characteristics[a]	Plasmid content	Virulence	Origin and comments
Shigella flexneri					
M25-8[b]	1b	*nad* Sul^r	1 large + small cryptic plasmids	+	Walter Reed Collection
M25-8A	1b	*nad* Sul^r	Small cryptic plasmids	−	Previous work (19)
M25-8AMinI	1b	*nad* Sul^r	Small cryptic plasmids	−	This work; NMG[c] mutant of M25-8A (minicell producer)
M25-8A(pWR110)	1b	*nad* Sul^r Kan^r	pWR110 + small cryptic plasmids	+	Previous work (19); pWR110 mobilized from M90T(pWR110)
M25-8A (pWR110)MinI	1b	*nad* Sul Kan^r	Same as M25-8A(pWR110)	+	This work; NMG mutant of M25-8A(pWR110) (minicell producer)
J17B	3a	*nad*	1 large + small cryptic plasmids	+	Walter Reed Collection
J17BMinV	3a	*nad*	Same as J17B	+	This work; NMG mutant of J17B (minicell producer)
M90T	5	*nad*	1 large + small cryptic plasmids	+	Walter Reed Collection
M90T(pWR110)	5	*nad* Kan^r	pWR110 + small cryptic plasmids	+	Tn*5* inserted into large plasmid (19)
M90TMinI	5	*nad*	1 large + small cryptic plasmids	+	This work; NMG mutant of M90T (minicell producer)
M90TMinII	5	*nad* Kan^r	pWR110 + small cryptic plasmids	+	This work; NMG mutant of M90T(pWR110) (minicell producer)
M90TMinII-5	5	*nad*	pWR110 ΔTn*5* + small cryptic plasmids	−	This work; spontaneous deletion in pWR110 of M90TMinII
M90TMinII-1	5	*nad*	Small cryptic plasmids	−	This work; spontaneous loss of pWR110 from M90TMinII
M90T-98	5	*nad* Kan^r	1 large + small cryptic plasmids	−[d]	This work; Tn*5* inserted into large plasmid inducing microdeletion
M90T-98MinI	5	*nad* Kan^r	Same as M90T-98	−[d]	This work; NMG mutant of M90T-98 (minicell producer)
S. sonnei					
482-79I	Form I	*nad* Sul^r Str^r	1 large + small cryptic plasmids	+	Institut Pasteur Collection
482-79MinI	Form I	*nad* Sul^r Str^r	Same as 482-79I	+	This work; NMG mutant of 482-79I (minicell producer)
482-79II	Form II	*nad* Sul^r Str^r	Small cryptic plasmids	−	Institut Pasteur Collection

TABLE 1—*Continued*

Strain	Serotype	Relevant characteristics[a]	Plasmid content	Virulence	Origin and comments
482-79IIMinI	Form II	*nad* Sul^r Str^r	Same as 482-79II	−	This work; NMG mutant of 482-79II (minicell producer)
53GI	Form I	*nad*	1 large + small cryptic plasmids	+	Walter Reed Collection
9774I	Form I	*nad* Str^r	1 large + small cryptic plasmids	−	Walter Reed Collection
Escherichia coli					
4608-58	O143	None	1 large plasmid	+	Walter Reed Collection
4608-58A$_4$	O143	None	No plasmids	−	This work; avirulent derivative of 4608-58
4608-58MinVI	O143	None	1 large plasmid	+	This work; NMG mutant of 4608-58 (minicell producer)
4608-58A$_4$MinI	O143	None	No plasmids	−	This work; NMG mutant of 4608-58A$_4$ (minicell producer)

[a] *nad*, Requirement for nicotinic acid; Sul^r, Kan^r, and Str^r, resistance to sulfonamides, kanamycin, and streptomycin, respectively.
[b] Walter Reed strain number
[c] NMG, *N*-Methyl-*N'*-nitro-N-nitrosoguanidine.
[d] Cultures unable to evoke keratoconjunctivitis but invasive in HeLa cell model.

than 1 vegetative cell per 10^6 minicells.

Labeling of minicells and envelope preparation. Suspensions of minicells were labeled with [^{35}S]methionine essentially as described by Levy (2, 13). Briefly, methionine assay medium (Difco) was diluted to 25% concentration with minimal glucose salts medium (1) supplemented with 10 μg each of nicotinic and aspartic acids per ml in addition to 1,000 μg of ampicillin per ml. Purified minicells were suspended in this medium and incubated for 1 h at 37°C with aeration. [^{35}S]methionine (20 μCi/ml, 7,900 μCi/mmol; New England Nuclear Corp., Boston, Mass.) was added, and the cell suspension was incubated for 4 h. Labeled minicells were then pelleted by centrifugation for 10 min at 20,000 × *g* and resuspended in 0.05 M Tris-hydrochloride (pH 7.8) with 10% glycerol, and phenylmethylsulfonyl fluoride (Boehringer Mannheim GmbH, Mannheim, W. Germany), was added at 5.0×10^{-4} M. The minicells were ruptured by three passages through a French pressure cell (25,000 lb/in^2), and the envelope fraction was collected by centrifugation at 100,000 × *g* for 30 min. Cytoplasmic membrane was solubilized by two successive washes (10 min at 25°C) with 2.0% Triton X-100 in 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer at pH 7.4 (22). The remaining particulate outer membrane fraction was suspended in 0.05 M Tris-hydrochloride (pH 7.8), and total membrane-bound radioactivity was ascertained by counting a portion of this suspension in scintillation fluid (PCS II; Amersham, Arlington Heights, Ill.). The remaining suspension was centrifuged at 100,000 × *g* for 30 min, and the pellet was suspended at 2.0×10^4 cpm/μl in 0.06 M Tris-hydrochloride (pH 6.8) with 2.0% SDS and 1 mM EDTA. The sample was then dispensed in individual tubes (10 μl per tube) and frozen at −70°C.

Labeling and lysis of vegetative cells. *S. flexneri* 5 strain M90TMinI was grown for 18 h on Trypticase Soy agar at 37°C. The culture was harvested and suspended at an absorbance of 1.0 at 530 nm in a medium of 70% minimal glucose salts supplemented with 10 μg each of nicotinic and aspartic acid per ml, 25% methionine assay medium, and 5% Luria broth consisting of 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and 2 g of glucose per liter. [^{35}S]methionine was added to 20 μCi/ml, and the bacterial suspension was incubated for 4 h at 37°C. The culture was then pelleted by centrifugation and washed in 0.05 M Tris-hydrochloride (pH 7.8). The washed cells were lysed by boiling for 5 min in Tris-hydrochloride (0.06 M, pH 6.8) containing 2.0% SDS and 8.0% 2-mercaptoethanol. Lysates containing 5.0×10^5 cpm were dispensed in individual tubes and frozen at −70°C.

Polyacrylamide gel electrophoresis and fluorography. Protein samples were thawed, and 10 μl of Tris-hydrochloride buffer (0.06 M, pH 6.8), containing 2.0% SDS, 8.0% 2-mercaptoethanol, 40.0% glycerol, and 4.0% saturated bromophenol blue, was added. The sample tubes were boiled for 10 min, and the polypeptide components were separated by slab gel electrophoresis by the method of Laemmli (12). The gels were 13.0% acrylamide (acrylamide–*N*,*N'*-methylene-bisacrylamide, 30:0.8), and they were electrophoresed at a constant current of 15 mA.

After electrophoresis was completed, the gels were fixed overnight at 25°C in an aqueous solution of 10% (wt/vol) trichloroacetic acid, 10% glacial acetic acid, and 30% methanol. Fixed gels were impregnated with

an autoradiography enhancer (En^{3}Hance, New England Nuclear Corp.) for 1 h, fluors were precipitated by soaking the gels in an aqueous solution of 10% glycerol for 1 h, and the gels were dried onto a filter paper backing. The dried gels were exposed to Kodak Blue Brand film for 48 h at −70°C.

Electron microscopy. HeLa cell monolayers were grown on 35-mm plastic tissue culture dishes, and 2 ml of Eagle minimal essential medium containing approximately 1.0×10^9 minicells per ml was added. These dishes were centrifuged for 15 min in a Sorvall GLC-2B centrifuge (Ivan Sorvall, Norwalk, Conn.) at 4,000 rpm, and they were incubated for 30 min at 37°C in 5% CO_2. The monolayers were then fixed for 1 h at 4°C in a mixture of 2.5% glutaraldehyde and 2.0% osmium tetroxide (1:2) prepared in 0.01 M phosphate-buffered saline at pH 7.2. The fixed monolayers were washed three times in cold phosphate-buffered saline, and 1.0 ml of 1.0% colloidal thorium (Polysciences, Inc., Warrington, Pa.) in 3.0% acetic acid was added. After 24 h at 25°C, the monolayers were washed three times with phosphate-buffered saline, dehydrated in ethanol, and embedded in Spurr epoxy plastic (Polysciences, Inc.). Polymerization was initiated by incubation of infiltrated specimens overnight at 70°C, and the material was sectioned on an Ultratome V (LKB, Rockville, Md.). Sections were mounted on 300-mesh copper grids, stained in 2.0% methanol uranyl acetate for 10 min, rinsed extensively in distilled water, counterstained in lead citrate (Polysciences, Inc.) for 5 min, and again rinsed in distilled water. Stained preparations were examined in a JEOL-C electron microscope operated at 80 kV.

RESULTS

Molecular comparison of shigella and *E. coli* virulence plasmids. The virulence-associated plasmids of *S. flexneri* and *S. sonnei* are of similar molecular weight (17–19). This similarity suggested that a family of related plasmids could be associated with the invasive phenotype in these species. Therefore, restriction analysis was performed upon the 140-Mdal plasmids of *S. flexneri* serotypes 1, 3, and 5. The intact plasmids are shown in lanes A, B, and C of Fig. 1. Although each plasmid preparation yielded 19 *Eco*RI cleavage fragments, only 5 of these migrated at the same rate (lanes G, H, and I). When digested with *Bam*HI, the *S. flexneri* plasmids yielded 11 fragments, 3 of which migrated at the same rate (lanes N, O, and P).

The large plasmid species of three *S. sonnei* strains were also subjected to restriction analysis. The intact plasmids are shown in Fig. 1, lanes D, E, and F. The pattern of fragments from these plasmids suggested more homology than was apparent in the *S. flexneri* plasmids. For example, 18, 19, and 13 *Eco*RI cleavage fragments are visible in lanes J, K, and L, respectively, and 10 of these fragments migrated at the same rate. Four *Eco*RI fragments of approximately 11.5, 6.5, 5.5, and 2.6 Mdal were consistently found in *S. flexneri* and *S. sonnei* plasmids isolated from virulent strains. When digested with *Bam*HI, the series of *S. sonnei* plasmids yielded 9, 10, and 7 fragments (lanes Q, R, and S). Six of these fragments migrated at the same rate. Treatment of virulence plasmids with *Bam*HI yielded fewer fragments than with *Eco*RI, indicating that the *Bam*HI restriction sites were widely spaced. The small amount of interspecies homology suggested by *Eco*RI digestion was not apparent in these *Bam*HI fragments.

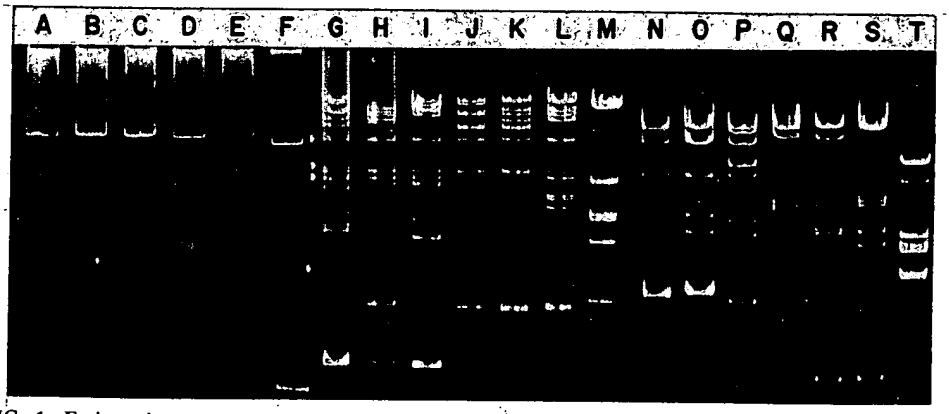

FIG. 1. Endonuclease restriction analysis of *S. flexneri* and *S. sonnei* virulence plasmids: agarose gel electrophoretic profiles of plasmid DNA obtained from (A) *S. flexneri* M25-8; (B) *S. flexneri* J17B; (C) *S. flexneri* M90T; (D) *S. sonnei* 53GI; (E) *S. sonnei* 482-79I; (F) *S. sonnei* 9774I. (G–L) *Eco*RI endonuclease cleavage of virulence plasmids displayed as in (A–F); (M) *Eco*RI cleavage of λ phage; (N–S) *Bam*HI endonuclease cleavage of virulence plasmids displayed as in (A–F); and (T) *Bam*HI cleavage of λ phage. Some of the thick bands seen amid restriction patterns correspond to uncleaved small cryptic plasmids. Arrows identify fragments of 11.5, 6.5, 5.5, and 2.6 Mdal in the *Eco*RI restriction pattern.

Since restriction analysis did not conclusively show that the *S. flexneri* and *S. sonnei* plasmids are closely related, these plasmids were further characterized by nitrocellulose filter blot hybridization. Radiolabeled plasmid DNA from *S. flexneri* strain M90T was used as a probe to identify homologous sequences in blotted *Eco*RI fragments from other virulence-associated plasmids. Figure 2, lane A, shows hybridization to the 140-Mdal plasmid of *E. coli* 4608-58. The digest of this plasmid is shown in Fig. 3, lane O. Figure 2, lane B, shows hybridization to fragments of *S. sonnei* 482-79I which correspond to those seen in lane K of Fig. 1. Lane C in Fig. 2 shows hybridization to homologous plasmid DNA from strain M90T, and lanes D and E show hybridization to *S. flexneri* serotypes 3 (JI7B) and 1 (M25-8). The *Eco*RI digests of these plasmids can be seen in Fig. 1, lanes I, H, and G. Hybridization of the ^{32}P-labeled plasmid DNA from *S. flexneri* 5 with restriction fragments from the other virulence-associated plasmids indicates that these plasmids do indeed constitute a closely related family.

Characterization of plasmids in strains of *Shigella* and invasive *E. coli* selected for biosynthetic molecular studies. Three sets of strains were selected for study of plasmid-encoded polypeptides. The plasmid profiles of these strains are shown in Fig. 3. Lanes A through G represent various *S. flexneri* strains, H and I are *S. sonnei* strains, and J and K are *E. coli* strains. Of the *S. flexneri* strains, lanes A and B show the plasmid content of minicell-producing strains M90TMinI and M90TMinII, respectively. The latter strain carried the Tn*5* transposon in a 140-Mdal plasmid, pWR110, described previously (21). Both strains were fully virulent. *S. flexneri* M90T-98MinI (lane C) also carried the Tn*5* transposon on a 140-Mdal plasmid; however, this strain was unable to induce keratoconjunctivitis in the guinea pig eye while retaining the ability to invade HeLa cells. Comparing the *Eco*RI restriction profiles of plasmids from M90TMinII (lane M) and M90T-98MinI (lane N) reveals the loss of two fragments and the appearance of one new fragment in the latter strain. Apparently the transposition process was accompanied by a small deletion in M90T-98MinI, but this deletion did not negate the invasive phenotype. Two kanamycin-sensitive variants of M90TMinII, isolated after serial passage at 42°C, were avirulent in both the Sereny test and the HeLa cell assay. One of these, M90TMinII-5 (lane D), was a strain which harbored a deleted form of pWR110. The *Eco*RI restriction profile of this plasmid (shown in lane N) indicated the loss of 10 fragments. Lane E of Fig. 3 shows that an autonomous pWR110 plasmid was not present in the other kanamycin-sensitive variant, M90TMinII-1. Lane F shows M25-8AMinI, an avirulent variant of *S. flexneri* 1 which no longer harbors an autonomous 140-Mdal plasmid, and lane G shows a virulent transconjugant of this strain which was constructed by mobilization of pWR110 from M90T(pWR110) with the Inc.F1 plasmid R386 as described previously (20).

The second set of strains comprise two minicell-producing isolates of *S. sonnei*. Strain 482-79MinI (Fig. 3, lane H) was a virulent strain which expressed the form I antigen, and 482-79IIMinI (lane I) was an avirulent form II derivative which had lost the virulence plasmid previously described (19). The third group of strains consisted of minicell-producing mutants of *E. coli* 4608-58, an invasive "shigella-like" strain. The virulent strain harbored a 140-Mdal plasmid (lane J), whereas an avirulent strain, which was isolated from an old culture, did not exhibit this plasmid (lane L). Lane P shows the 22 *Eco*RI digestion fragments from the large plasmid of virulent strain 4608-58MinVI.

Invasion of HeLa cells by *Shigella* minicells. The study of plasmid-encoded virulence determinants in purified minicells was based on the assumption that these anucleate bacterial cells retain the virulence phenotype of vegetative parental cells. Since minicells are incapable of multiplication, in vivo virulence assays such as

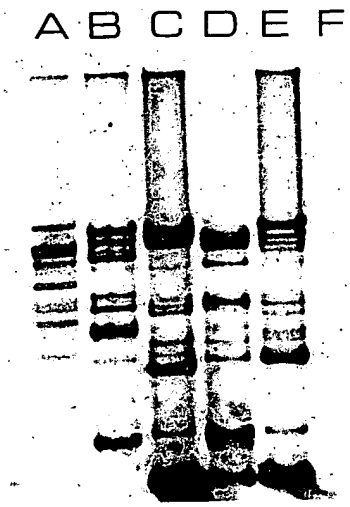

FIG. 2. Detection of sequence homology in *Eco*RI restriction fragments from plasmids of enteroinvasive species. Digested plasmid DNA was separated by agarose gel electrophoresis, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled plasmid DNA from *S. flexneri* 5 strain M90T. Autoradiography was used to detect homologous sequences in plasmids from (A) *E. coli* 4608-58, (B) *S. sonnei* 482-79I, (C) *S. flexneri* M90T, (D) *S. flexneri* J17B, (E) *S. flexneri* M25-8, and (F) λ phage.

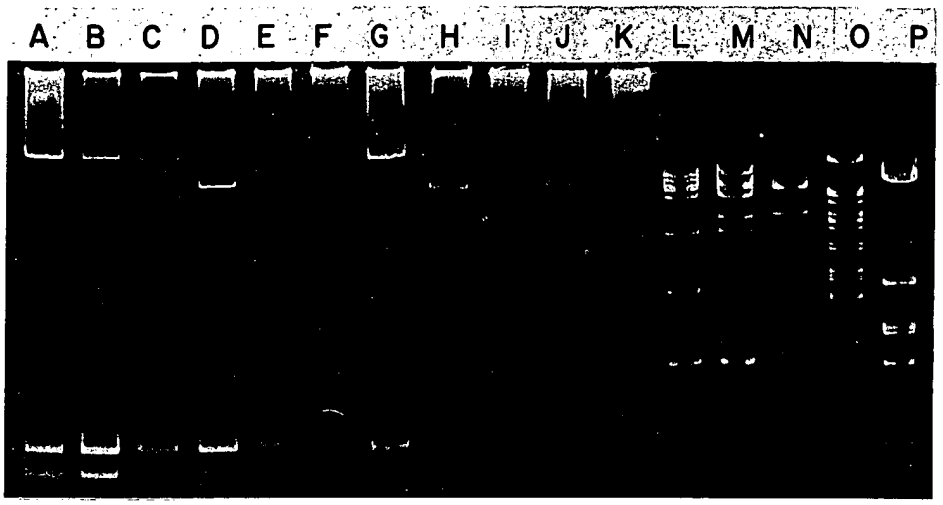

FIG. 3. Agarose gel electrophoretic profiles of *Shigella* and invasive *E. coli* virulence plasmids harbored by minicell-producing strains selected for outer membrane protein studies: (A) *S. flexneri* M90TMinI; (B) *S. flexneri* M90TMinII; (C) *S. flexneri* M90T-98MinI; (D) *S. flexneri* M90TMinII-5; (E) *S. flexneri* M90TMinII-1; (F) *S. flexneri* M25-8AMinI; (G) *S. flexneri* M25-8A(pWR110)MinI; (4) *S. sonnei* 482-79MinI; (I) *S. sonnei* 482-79IIMinI; (J) *E. coli* 4608-58MinVI; (K) *E. coli* 4608-58A_4MinI. *Eco*RI endonuclease cleavage patterns of plasmids from (L) *S. flexneri* M90TMinII, (M) *S. flexneri* M90T98MinI, (N) *S. flexneri* M90TMinII-5, (O) *E. coli* 4608-58MinVI, and (P) λ phage DNA.

the Sereny test are inappropriate. Therefore, minicell suspensions were tested for virulence in the HeLa cell model. Figure 4A shows an electron micrograph of a typical HeLa cell 30 min after exposure to minicells isolated from a culture of *S. flexneri* M90TMinI. Many osmophilic minicells can be seen within membrane-bound vacuoles in the cytoplasm of this cell. Figure 4B shows a higher magnification of the process of minicell invasion. Three cell-associated minicells are seen in different stages of infection. One minicell is attached to the surface of the HeLa cell, and two microvilli with prominent microfilaments are seen in close proximity to this bacterium. Another minicell is apparently enclosed within a membranous vacuole, but the presence of thorium particles around this bacterium and around the HeLa cell membrane indicates that this minicell has not been completely engulfed. A third minicell can be seen in a membrane-bound vacuole which is free of thorium particles. This is an organism which has been endocytosed and is completely enclosed by host cell cytoplasm (9). Figure 4C shows minicells purified from a culture of M90MinII-1 which had lost the 140-Mdal plasmid (see Fig. 2, lane E). Even though these minicells were centrifuged onto the surface of the HeLa cell monolayer and incubated for 2 h, they did not establish intimate contact with the plasma membrane. This behavior is consistent with the avirulent phenotype of the vegetative cells of this strain.

Plasmid-mediated synthesis of outer membrane proteins. Figure 5, lane B, shows the labeled polypeptides contained in a vegetative cell lysate of *S. flexneri* 5 (strain M90TMinI). These cells had been grown in the presence of [^{35}S]methionine, and the labeled components represent both chromosomal and plasmid transcripts. Lane C shows the labeled polypeptides in a lysate of minicells isolated from M90TMinI. Of the many polypeptides labeled in vegetative cells, only 15 polypeptides were detectable in minicells which had been incubated with [^{35}S]methionine. Since these minicells contained only plasmid DNA (5), newly synthesized proteins which had incorporated radiolabeled precursors presumably represent plasmid-coded products. Almost all of the plasmid-coded polypeptides copurified with the outer membrane fraction (lane D), so plasmid expression was subsequently studied in the outer membrane fraction of minicells.

Plasmid DNA from small "cryptic" plasmids would be transcribed in minicells, and post-translational incorporation of methionine into proteins having basic NH_2-terminal amino acids has been reported (21), so residual labeling of polypeptides was analyzed in syngenetic minicells which did not harbor a large, virulence-associated plasmid. For example, lane A in Fig. 6 shows that one polypeptide of 56×10^3 daltons (56 K) was labeled in the outer membrane of avirulent *S. sonnei* strain 482-79IIMinI, whereas

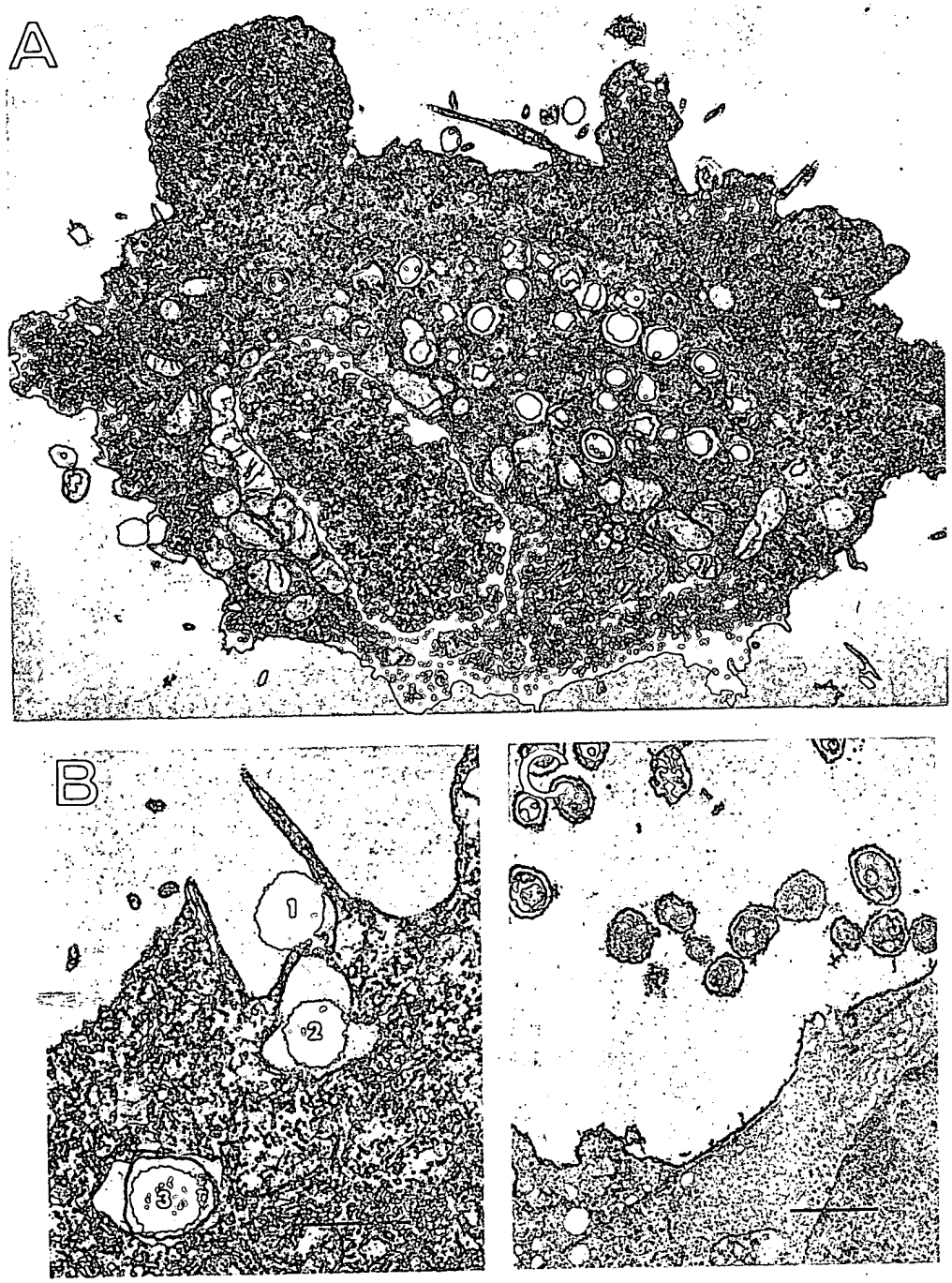

FIG. 4. Transmission electron micrographs of HeLa cells exposed to *S. flexneri* 5 minicells for 30 min. (A) HeLa cell infected with strain M90TMinII. (B) Two attached M90TMinII minicells (1, 2) and one intracellular minicell (3). (C) Extracellular M90TMinII-1 minicells. Bar, 1.0 μm.

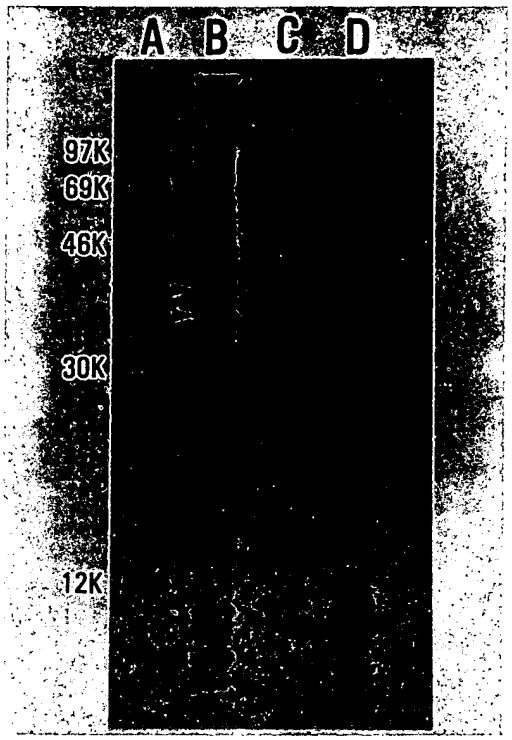

FIG. 5. Electrophoretic separation of polypeptides of radiolabeled *S. flexneri* M90TMinI after incubation with [^{35}S]methionine for 4 h. (A) Molecular weight markers; (B) lysate of vegative cells and minicells (arrows indicate major outer membrane proteins); (C) lysate of minicells; (D) outer membrane fraction of minicells.

12 polypeptides were labeled in the outer membrane of minicells which harbored the 120-Mdal virulence-associated plasmid (482-79MinI, lane B). Nine polypeptides, ranging from 12K to 62K, were consistently associated with the invasive phenotype in *S. flexneri* serotypes 3 (J17BMinV, lane C) and 5 (lanes D, E, and F) and in *E. coli* (4608-58MinVI, lane K). In contrast, seven polypeptides which were labeled in virulent strains of *S. flexneri* 5 were not labeled in an avirulent strain which had sustained a large deletion in the 140-Mdal plasmid (M90TMinII-5, lane F). Lanes H and I of Fig. 6 show the residual labeling of polypeptides in strains of *S. flexneri* serotypes 5 and 1 which no longer harbored an autonomous 140-Mdal plasmid. The avirulent serotype 1 strain was restored to virulence upon the conjugal transfer of the 140-Mdal plasmid, pWR110, from *S. flexneri* 5. Lane J shows that this transconjugant, M25-8A(pWR110)MinI, expressed a complement of plasmid-coded polypeptides similar to that expressed by an *S. flexneri* 5 strain which harbored pWR110 (M90TMinII; Fig. 6, lane F).

DISCUSSION

It is now well established that extrachromosomal elements are involved in the virulence of *S. sonnei* (19) and *S. flexneri* (20). The 120-Mdal plasmid of *S. sonnei* is necessary for expression of LPS O-polysaccharide side chains, whereas no LPS biosynthetic activity has been attributed to the 140-Mdal plasmid of *S. flexneri*. Obviously these two plasmid species are not identical, but they may share a common origin which would be reflected in homologous base sequences. Therefore, two restriction endonucleases (*Eco*RI and *Bam*HI) were employed in the analysis of the virulence-associated plasmids of *S. flexneri* (serotypes 1, 3, and 5), *S. sonnei*, and an enteroinvasive strain of *E. coli*. Whereas the majority of *S. sonnei* plasmid restriction fragments migrated at the same rate, indicating highly homologous sequences, only a few restriction fragments from *S. flexneri* and *E. coli* virulence plasmids showed identical molecular size. However, blotting-hybridization studies using a ^{32}P-labeled *S. flexneri* 5 plasmid probe demonstrated that all of these virulence-associated plasmids share homologous sequences distributed throughout the molecule. These data suggest that the large plasmids of *S. flexneri*, *S. sonnei*, and enteroinvasive *E. coli* are derived from a common ancestor. These plasmids have apparently evolved independently within the different enteroinvasive species and serotypes while undergoing microevolutionary mutations leading to variations in restriction sites. Since the pWR110 plasmid harbored by *S. flexneri* strain M90T restores the virulence of other *S. flexneri* serotypes (20) and of enteroinvasive *E. coli* (18), the primal plasmid virulence genes have evidently been conserved.

The observed association of plasmids with virulence in invasive enteric pathogens stimulated investigation of plasmid-mediated biosynthetic activity. Plasmid gene products may indirectly contribute to virulence by encoding enzymes for the synthesis of such constituents as the form I LPS antigen of *S. sonnei*, which increases bacterial resistance to host defense systems. However, there are also many examples of plasmid-coded structural proteins which are directly responsible for phenotypic alterations (14–16). Since the initiation of epithelial cell infection involves cell-cell interactions, it is possible that plasmid-coded proteins inserted into the outer membrane of invasive organisms may function as virulence determinants.

Proteins can best be identified as plasmid-encoded by radiolabeling newly synthesized

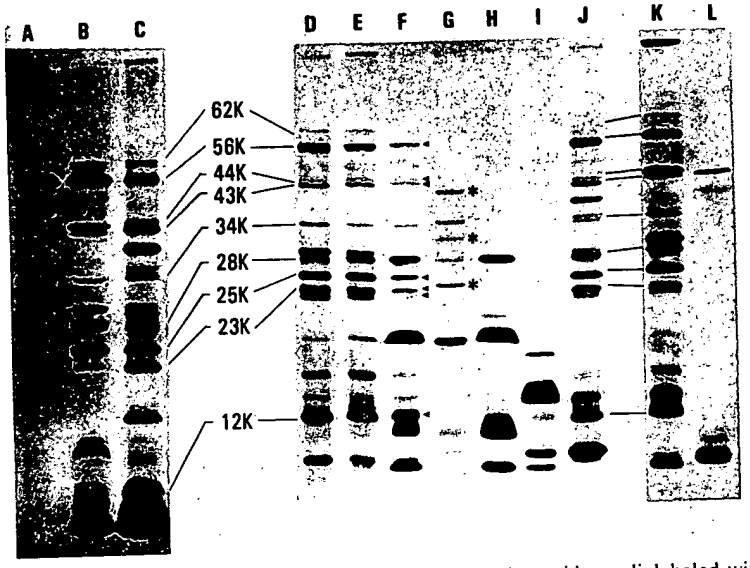

FIG. 6. Electrophoretic separation of minicell outer membrane polypeptides radiolabeled with [^{35}S]methionine. (A) *S. sonnei* 482-79IIMinI; (B) *S. sonnei* 482-79I; (C) *S. flexneri* J17BMinV; (D) *S. flexneri* M90TMinI; (E) *S. flexneri* M90T-98MinI; (F) *S. flexneri* M90TMinII; (G) *S. flexneri* M90TMinII-5; (H) *S. flexneri* M90TMinII-1; (I) *S. flexneri* M25-8AMinI; (J) *S. flexneri* M25-8A(pWR110)MinI; (K) *E. coli* 4608-58MinVI; and (L) *E. coli* 4608-58A$_4$MinI. Polypeptides which were consistently labeled in minicells from invasive strains are identified by molecular weight designations, and arrows indicate polypeptides which were labeled in M90TMinII but not in M90TMinII-5. Asterisks identify new polypeptides labeled in the latter strain.

polypeptides in anucleate minicells which contain only plasmid DNA (5). Recently, minicell-producing strains of virulent shigellae were isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, but it was concluded that minicells from these strains were noninvasive (6). In contrast we found that, after centrifugation, *Shigella* and *E. coli* minicells which harbored the virulence plasmid readily invaded HeLa cell monolayers. Whereas syngenetic plasmid-free minicells were noninvasive. Since minicells containing the virulence plasmid retained the determinants necessary for infection of mammalian cells, they were deemed appropriate vehicles for the study of plasmid expression. Purified suspensions of these cells were incubated with [^{35}S]methionine, and plasmid-encoded polypeptides were identified by fluorography of SDS-polyacrylamide gels.

A complement of nine polypeptides, ranging from 12K to 64K, was labeled in the outer membrane of all minicells which harbored virulence-associated plasmids. A noninvasive *S. flexneri* strain (M90TMinII-5), which had suffered a large deletion in the pWR110 plasmid, no longer incorporated labeled amino acids into seven of these polypeptides. However, three new polypeptides were labeled in this mutant. These may represent truncated transcripts or unprocessed precursor polypeptides. Study of additional deletion mutations may allow identification of a minimum complement of plasmid-coded polypeptides which is consistent with the invasive phenotype. It should be emphasized, however, that a causal relationship has not been established between expression of plasmid-coded outer membrane proteins and the invasive phenotype. In addition, the mechanism by which these proteins induce uptake of bacteria is a matter of conjecture. It has been proposed that they are constituents of bacterial receptors which sequentially bind to unidentified determinants on the surface of host cells (T. L. Hale, P. A. Schad, and S. B. Formal, Med. Microbiol., in press). Testing of this hypothesis offers a promising approach to the study of the molecular basis of tissue invasion by bacterial agents.

The similarity of plasmid-coded polypeptides in invasive strains of *S. flexneri*, *S. sonnei*, and *E. coli* is consistent with the DNA sequence homology found in the plasmids of these strains. Identification of plasmid restriction fragments which encode the determinants of the invasive phenotype may allow cloning and amplification of these determinants in avirulent recipients. Such recombinant strains might have potential as live vaccine candidates, or amplified virulence determinants might be isolated from clones and incorporated into a cell-free vaccine. Thus continued analysis of both the genetics of

virulence and the products encoded by virulence genes is essential for the rational development of effective vaccines against invasive enteric pathogens.

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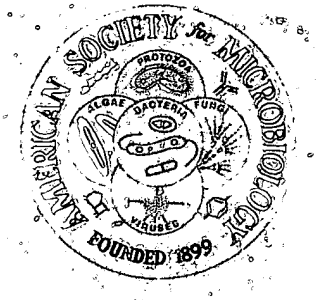

It is characteristic of Science and Progress that they constantly open new fields of vision. PASTEUR

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